INTRAMOLECULAR HETEROGENEITY OF MITOCHONDRIAL AND CHLOROPLASTIC DNA

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SUMMARY

Mitochondrial and chloroplastic DNA from unicellular and higher organisms are splitted into several bands by $Cs_2 SO_4$ density gradient ultracentrifugations in the presence of Ag^+ ions. The banding pattern includes a main band DNA, a heavy satellite DNA and, in the most cases, a light satellite DNA. The buoyant densities of these Ag^+ satellite DNAs appear similar for the various mitochondrial and chloroplastic DNAs investigated and fit well the two classes of Ag^+ satellite DNAs found in nuclear and total DNAs of various organisms. The heterogeneity of organelle's DNA is discussed.

INTRODUCTION

Discrepancy between analytical and kinetic complexity (1, 2) has revealed the occurence of repeated sequences in chloroplastic DNA (Euglena, 3, and lettuce, 4) and in mitochondrial DNA (Tetrahymena, 5, chick embryo, 6, and lettuce, 4). The nature of these reiterated sequences is not clear yet, since the reiteration can result from the presence of numerous identical molecules per organelle, as recently shown in Euglena (several dozen circles per chloroplast, 7) and from intramolecular heterogeneity as well (4). On the other hand, buoyant density shifts induced by the binding of homopolyribonucleotides to denatured mitochondrial DNA have detected the occurence of identical nucleotides clusters in chick (6) and in Tetrahymena (8) ; such regions

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are also likely present in the mitochondrial DNA from ρ^- mutants of yeast (9). Recently, intramolecular heterogeneity of Chlamydomonas chloroplastic DNA has been deduced from melting profiles (10, 11). It must be emphasized that mitochondrial DNA, like chloroplastic DNA, forms a single and symetrical band when analysed by CsCl density-gradient.

The techniques mentioned above did not lead to the separation of the reiterated sequences. We report here that mitochondrial and chloroplastic DNAs are splitted up into several families of molecules by Cs_2SO_4 density gradient in the presence of Ag^+ ions. Both unicellular and higher organisms were investigated.

MATERIAL AND METHODS

Mitochondrial fractions from cotyledons of etiolated cucumber seedlings (12)-germinated in darkness at 28°C for 5 days- were prepared according essentially to Wells and Birnstiel (4). All operations were carried out at 0-4°C. The material was carefully washed and homogenized with a pestle in a chilled mortar in 3ml/g fresh mat. of 0.3 M sucrose, 5.10^{-2}M Tris-HCl, 3.10^{-3}M $CaCl_2$ and 0.1% (w/v) bovine serum albumin, adjusted to pH 7.2. The homogenate was filtered through six layers of gauze and the filtrate centrifuged at 500 g for 10 min to remove nuclei, starch and cell debris. The supernatant was centrifuged at 3,000 g for 10 min to sediment etioplasts and further starch grains and cell debris. The mitochondrial fraction was obtained from the 3,000g supernatant by centrifuging at 20,000 g for 15 min. The mitochondrial pellets were resuspended in the homogenization medium made 8 mM with respect to $MgCl_2$ and incubated with DNase (50 µg/ml) at 0°C for I hour to destroy extramitochondrial DNAs. The DNase action was stopped by the addition of 3 vol. of 0.15MNaCl, 0.1 M EDTA, pH 8.0. Mitochondria were collected by centrifugation and the DNA extracted as previously described (13), without spooling, and purified by DNase-free RNase and pronase (4).

Chloroplastic fractions were prepared from maize and spinach, all operations being achieved at 0-2°C. Leaves were carefully washed and ground in a chilled mortar with 4 ml/g fresh mat. of 0.3 M sucrose, 5.10^{-2} M Tris-HCl, 3.10^{-3} M EDTA, 0.1 % (w/v) bovine serum albumin, pH adjusted to 7.3. The homogenate was filtered through six layers of gauze and then through a nylon net (Blutex, 50 µ) ; the filtrate was centrifuged at 100 g for 5 min to remove nuclei and starch. The supernatant was centrifuged at 3,000 g for 10 min, the pellet resuspended in the homogenization medium and the chloroplastic fraction sedimented at 5,000 g for 7 min. The pellet was resuspended in 16 ml of homogenization medium

and 2 ml fractions were layered on 8 tubes containing discontinuous Ludox gradients (14) in the same medium (2,5%, 5%, 10%, 15%, 22%, 5 ml each). After spinning at 500 g for 15 min, the chloroplast banding was similar to that published by Lyttleton (14). Chloroplastic bands were harvested, received 2 vol. of 0.3 M sucrose, 5.10^{-2} M Tris-HCl, 8.10^{-3} M $MgCl_2$, 0.1% bovine serum albumin, pH 7.3 and were sedimented at 5,000 g for 7 min. After another washing in the same medium, they were DNase treated as mentioned above and the DNA extracted in the same conditions.

Algae chloroplastal DNA was isolated from synchronous Chlorella pyrenoïdosa (strain 211/8b, Göttingen) axenically grown according to Lörenzen (15). DNA was extracted from whole cells as previously described (16) and the chloroplastic satellite DNA isolated by several preparative CsCl gradients.

Yeast (wild) mitochondrial DNA, purified by preparative CsCl density-gradients was a generous gift from Dr. I. Fukuhara (C.G.M., CNRS, 91 - Gif/Yvette). All DNAs were extensively dialysed against several liters of 1 or 0.1 x SSC at 2°C. Analytical CsCl density-gradients contained 2 to 20 µg DNA plus 1.8 µg of 2C phage DNA as a marker (buoyant density 1.742 g/ml). Analytical Ag^+-Cs_2SO_4 (17) contained 20 to 50 µg in 0.1 M Na_2SO_4, enough 10^{-3} M $AgClO_4$ to give a Ag^+/DNA-P molar ratio of 0.35 (18), enough 0.1M borate buffer pH 9.2 to give a final concentration of 5.10^{-3} M and Cs_2SO_4 (Merck suprapur) to obtain a final density close to 1.525 g/ml. A few drops of FC 43 oil were added in the analytical cell to determine more accurately the bottom edge of the centerpiece for the Ag^+-Cs_2SO_4 runs. Ultracentrifugations were achieved in a Spinco model E with UV optics, at 44,000 rpm, 25°C, for at least 20 hours. Buoyant densities were calculated according to Vinograd and Hearst (19) for the Ag^+-Cs_2SO_4 ultracentrifugations, using the liquid density after the run and the limiting isoconcentration distance.

RESULTS and DISCUSSION

Mitochondrial DNA: cucumber mitochondrial DNA (Fig. 1-B), like yeast mitochondrial DNA (Fig. 2-A), forms a single and symetrical band in CsCl. When analysed by Ag^+-Cs_2SO_4 density-gradient, cucumber mitochondrial DNA (Fig. 1-C) is fractionated in a main band (1.510-1.520), a light satellite (1.455-1.465) and a heavy satellite (1.600-1.610). Yeast mitochondrial DNA gives a main band (1.525-1.535), two light satellites (1.420-1.430 and 1.445-1.455) ; a small shoulder occurs at 1.560.

Chloroplastic DNA: maize chloroplatic DNA (Fig. 3-A) like Chlorella chloroplastic DNA (Fig. 4-B) forms a single and symetrical band, even in heavy-loaded cell (Fig. 3-A). Maize chloroplastic DNA is fractionated by

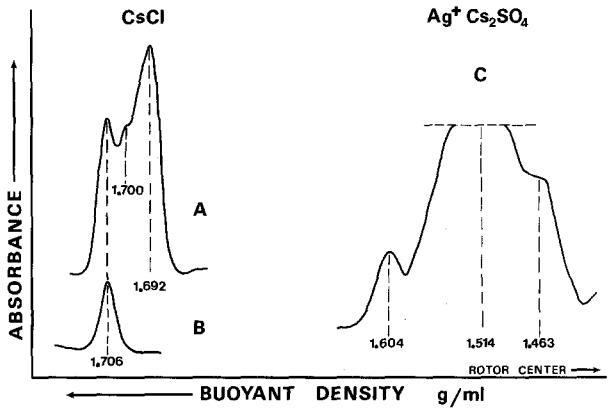

Fig. 1 : Microdensitometer tracings of the photographic records obtained after analytical density-gradient ultracentrifugations of mitochondrial DNA from etiolated cucumber cotyledons : A, whole cell DNA centrifuged in CsCl density-gradient shows a main band at 1.693 g/ml and a heavy satellite at 1.706 g/ml ; a shoulder is visible at 1.700 g/ml (marker DNA not shown) ; B, DNA prepared from the purified mitochondrial fraction, centrifuged in CsCl density-gradient, gives a unimodal band at 1.706 g/ml (marker DNA not shown) ; C, mitochondrial DNA (55 µg) analysed by Ag^{+}-Cs_2SO_4 density gradient.

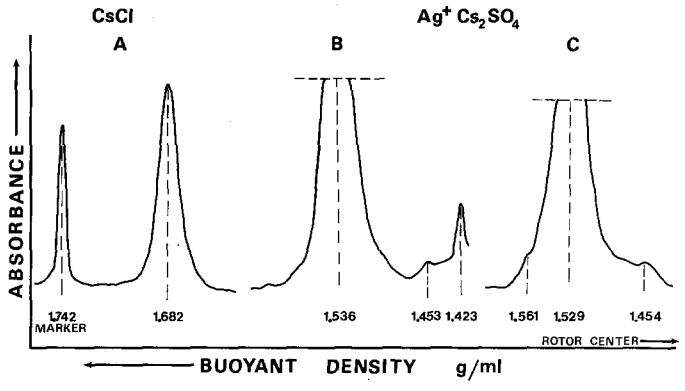

Fig. 2 : microdensitometer tracings of the photographic records obtained after analytical density-gradient ultracentrifugations of yeast mitochondrial DNA ; A, mitochondrial DNA (6 µg) centrifuged in CsCl density-gradient gives a unimodal band at 1.682 g/ml ; the nuclear DNA usually bands at 1.699 g/ml ; B, mitochondrial DNA (35 µg), Sw_{20} = 16 s) analysed by Ag^{+}-Cs_2SO_4 density-gradient ; C, same as B, after a mild shearing.

Ag^{+}-Cs_2SO_4 (Fig. 3-B) in a main band (1.520-1.540) with a light satellite (1.450-1.470) and a heavy satellite (1.590-1.610) ; the profile being rather similar to that of cucumber mitochondrial DNA. Chlorella chloroplastic DNA exhibits a striking heterogeneity with a main band (1.570-1.580) and an important heavy satellite (1.630-1.640). The gradient does not reveal light satellite, but the amount of DNA (25 µg) centrifuged

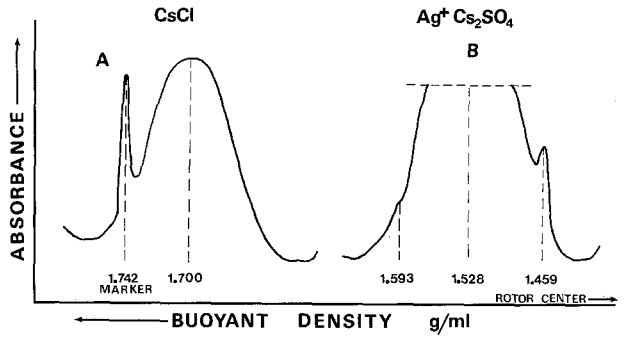

Fig. 3 : Microdensitometer tracings of the photographic records obtained after analytical density-gradient ultracentrifugations of maize chloroplastic DNA : A, chloroplastic DNA (20 µg) centrifuged in CsCl density-gradient, gives a unimodal band at 1.700 g/ml ; although heavy loaded, the gradient does not detect any shoulder/satellite (like Cs_2SO_4 gradients without Ag^+) ; B, chloroplastic DNA (35 µg) analysed by Ag^+-Cs_2SO_4 density-gradient.

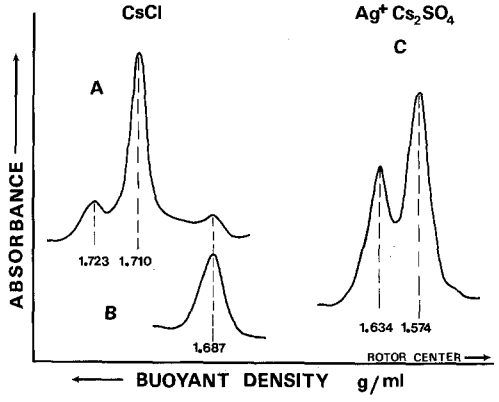

Fig. 4 : Microdensitometer tracings of the photographic records obtained after analytical density-gradient ultracentrifugations of chloroplastic DNA from Chlorella pyrenoïdosa : A, whole cell DNA, centrifuged in CsCl density-gradient, shows a main band at 1.710 g/ml, a heavy satellite at A.723 g/ml and a light satellite, of chloroplastic origin, at 1.687 g/ml ; B, the chloroplastic satellite DNA, purified by preparative CsCl density-gradients, analysed in CsCl gradient (marker DNA not shown) ; C, chloroplastic satellite DNA (25 µg) fractionated by Ag^+-Cs_2SO_4 density-gradient.

could be a limiting factor.

Intramolecular heterogeneity. The buoyant densities here reported are less accurate than the values obtained in classical Cs_2SO_4 and CsCl. They depend on the Ag^+/DNA-P molar ratio which is difficult to set sharply ; there is no possibility, until now to add a marker DNA, and using such heavy loaded cells likely disturbs the salt gradient. Nevertheless, several runs achieved on the same chloroplastic DNA solution as well as on another chloroplastic DNA (spinach) gave similar profiles.

Molecular nature of the DNA satellites. Ag^+ is known to bind specifically G-C base-pairs, the resulting shift in buoyant density being generally proportional to the mean G+C content. Although exhibiting homogenous (or Gaussian) behaviour in CsCl and Cs_2SO_4 density gradients, all the mitochondrial and chloroplatic DNA we have examinated are fractionated into several families of molecules by Ag^+. We must outline th discrepancies between the mean G+C content and the buoyant density shifts induced by Ag^+ and Hg^+ have already been reported for reiterated fractions of DNA : from human cells (total DNA, 20), mouse, guinea pig, calf (nuclea DNA, 21), tobacco and salsifis (total DNA , 22) and wheat (nuclear DNA, 2: The nature of these DNA fractionations by Ag^+ is not clear yet , several structural characteristics could be involved ; the linearity or circularity of the molecules can apparently be ruled out since electron microscope observations of mitochondrial and chloroplatic DNA (24) from higher plants reveals linear molecules only. The DNA molecular weight may affect the relative amount of the different families revealed by Ag^+, since the yeast mitochondrial DNA (Sw_{20} = 16), when removed from the analytical cell (Fig. 2-B), sheared by 3 passages through a 26 gauge needle, and respun without other treatment, shows a marked increase in the heavy shoulder occuring at 1.560 g/ml (Fig. 2-C). It seems unlikely that these Ag^+ satellite DNAs could represent partially denatured DNA, DNA-RNA hybrids and a fortiori RNA, since these structures band at higher buoyant densities in Cs_2SO_4 gradients without Ag^+ (25) ; anyhow they cannot explain the light Ag^+ satellite DNA.

Aside from these structural criteria, the most important factor involved in the Ag^+ fractionation seems to be the reiteration of nucleotides or sequences, as judged by the fact that all the Ag^+ DNA satellites have been shown to be highly reiterative (20 to 23). Strickingly, the buoyant densities of these Ag^+ satellite DNAs, obtained from various organisms (20 to 23), range into two classes, 1.43-1.47 and 1.54-1.60 (26). That the mitochondrial and chloroplastic Ag^+ satellite DNAs reported here fit well these two classes, whatever their origin and mean G+C content, appears very significant. These DNA fractions which bind Ag^+ in a different way than that described by Jensen et al (17) could represent peculiar nucleotidic sequences, common to all DNAs, and perhaps associated to peculiar genetical function.

Intramolecular heterogeneity of mitochondrial and chloroplastic DNA is demonstrated for unicellular and higher organisms ;further characterizations are required to clear the relationships between the different families of DNA molecules revealed by Ag^+ binding and the different components detected by kinetic complexity (4) and melting profiles (10).

Isolation of suitable amounts of the different Ag^+ satellite DNAs by preparative Ag^+-Cs_2SO_4 density gradients is under progress.

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